Site-dependent biological activity of valinomycin analogs bearing derivatizable hydroxyl sites

Cosimo Annese, Daniela I. Abbrescia, Lucia Catucci, Lucia D’Accolti, Nunzio Denora, Immacolata Fanizza, Caterina Fusco and Gianluigi La Piana

Valinomycin (VLM, 1) is a K⁺ ionophore cyclodepsipeptide capable of depolarizing mitochondria and inducing apoptosis to several mammalian cell types, including a number of tumor cell lines. With the aim of creating VLM-based ligand-targeted anticancer drugs that may selectively convey VLM to pathological cells, we have previously introduced derivatizable hydroxyl handles into the VLM structure, allowing to access a three-entity library of monohydroxyl VLMs (HyVLMs) bearing the OH group at the isopropyl side chain of a D-Hyi, D-Val, or L-Val residue (analogs 2–4, respectively). Herein, the levels of bioactivity retained by the conjugable HyVLMs have been assessed on the basis of their ability to alter the functionality of isolated rat-liver mitochondria. Experiments run with HyVLMs in the range 1–10 nM and in 20 or 125 mM KCl medium show that the hydroxyl group reduces the potency of HyVLMs relative to VLM to an extent that depends upon the molecular site involved in the hydroxylation. On the other hand, estimation of the stability constants of complexes (in methanol at 25 °C) of each analog with Na⁺, K⁺, and Cs⁺ reveals that HyVLMs nicely retain the VLM binding features, except for a moderate increase in the stability of Na⁺ complexes. These findings, along with pertinent structural considerations, suggest that the incorporation of OH into the VLM structure might actually have altered its K⁺ transporting ability across mitochondrial membranes. Besides facing new aspects of VLM structure–activity relationship, these studies set the basis for the rational design of ligand-HyVLMs conjugates through derivatization of hanging OH group. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: valinomycin; hydroxylation; ionophore; natural compound; mitochondria; cyclic depsipeptide; mitochondrial membrane potential

Introduction

Ionophores are a wide class of both synthetic and naturally occurring compounds capable of binding and passively moving cations across cellular membranes, thus bringing a perturbation in cellular cation homeostasis, which in several cases proves fatal [1,2]. As a consequence, ionophores are generally potent antimicrobial, antifungal, and antitumor agents, but their high toxicity and narrow therapeutic index have so far prevented their clinical employment. Nevertheless, recent investigations have pointed out the high therapeutic potential of some ionophores, thus marking a renewed interest in these compounds for human cancer therapy [3,4].

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In such a context, we have recently taken up the challenge of building ligand-targeted anticancer drugs on the basis of the potent and broad-spectrum antiproliferative activity of the K⁺ ionophore valinomycin (VLM, 1), a naturally occurring cyclo depsipeptide, chemically consisting of a three-repeating sequence of the tetramer D-α-hydroxyisovaleryl-L-valyl-L-lactyl-L-valyl (D-Hyi-D-Val-L-Lac-L-Val) cyclically arranged to form a 36-membered macro-ring (Figure 1) [1]. Such peculiar structure makes VLM act as a highly selective potassium ionophore, capable of depolarizing mitochondria, and trigger apoptosis [4–8]. Hence, its renowned antitumor activity [4,8], among others, ensues.

In spite of its enormous therapeutic potential, VLM has not so far been employed for clinical purposes, owing to its high levels of toxicity. As a consequence, new approaches are needed to better unravel the mechanisms by which VLM induces cell death in non-tumor cells and to optimize its therapeutic window.

In this context, we have recently developed a new class of VLM-based anticancer drugs, which we termed ligand-HyVLMs, by introducing derivatizable hydroxyl (OH) groups at the isopropyl side chain of the D-Hyi, D-Val, or L-Val residue of VLM (analogs 2–4, respectively). The OH group is conjugated to VLM through derivatization of the hydroxyl group at the angular position, which is conjugated by the use of a three-entity library of monohydroxylated analogs (HyVLMs).

The use of OH as a linkage site in the design of ligand-HyVLMs is based on the rationale that the OH group is a powerful electron-donor group, which, upon conjugation, may contribute to the formation of a new hydrogen bond network in the drug–target complex. This new hydrogen bond network may lead to a change in the conformation of the drug, thereby altering its affinity constant and, consequently, its biological activity. Moreover, the OH group is a versatile functional group that can be further derivatized to introduce different functional groups, such as carbonyl, amide, or ester groups, which may further modify the biological activity of the drug.

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of cytotoxicity [6,7]. If, on one hand, the metabolic engineering has set the stage for generating VLM structural analogs that could provide an alternative [9], the targeting of VLM to diseased cells through conjugation with directing ligands [10–12] might represent a potent strategy to increase its therapeutic potential. Studies in this direction, however, still appear at an embryonic stage [13] and are limited by the lack of suitable derivatizable ‘chemical handles’ (e.g. –NH2, –OH, –SH, and –COOH) in the molecular structure, which is clearly prerequisite for conjugation [10–12].

In the course of our studies on the selective hydroxylation of amino acids and peptides [14,15], we showed that the direct reaction of VLM with methyl(trifluoromethyl)dioxirane (TFDO) is a convenient route to introduce OH ‘handles’ in its frame [15], allowing to access a small library of three hydroxyl analogs (HyVLMs 2–4, Figure 1) bearing a OH group at the isopropyl side-chain of a β-Hyi, β-Val, or γ-Val residue (Figure 1).

As a part of our research program in this area, we address here the question of how the presence of a hydroxyl group as in analogs 2–4 (Figure 1) can affect the VLM bioactivity, in relation to its ionophore and ultimately pro-apoptotic properties. Thus, the impact of the OH group was evaluated on the basis of the effect of each HyVLM on bioenergetics parameters of isolated rat-liver mitochondria (RLM). Results clearly show that HyVLMs are less active than VLM, and the extent of diminished bioactivity reflects the site of hydroxylation. Nevertheless, we find that such analogs strictly retain the characteristics of cation binding of VLM.

**Materials and Methods**

**Materials**

Valinomycin (1) was purchased from Apollo Scientific (UK) and used as received in the preparation of HyVLMs 2–4, according to a procedure developed in our laboratories [15]. Products 1–4 used in cation binding and bioassay experiments were iteratively purified by preparative HPLC [column Ascentis® Si, 250 × 10 mm, 5 μm; eluent hexane/isopropanol] until >98% purity (HPLC) was attained. All other reagents were of analytical grade and mainly obtained from Sigma-Aldrich (Milan, Italy). All the solutions, with the exception of mitochondrial respiratory chain inhibitors and of compounds 1–4, which were dissolved in absolute ethanol, were prepared with quartz double distilled water.

**Isolation and Incubation of Mitochondria**

Rat-liver mitochondria were isolated in mannitol-sucrose medium as previously described [16], incubations were carried out at 25 °C in a standard sucrose medium at pH 7.4, consisting of 250 mM or 40 mM sucrose and 20 mM HEPES–Tris. This ‘zero-K+’ medium was chosen as the basal incubation medium, to which, depending on the experimental approach utilized, KCl was added to the desired concentration (20 or 125 mM). The oxidative phosphorylation efficiency was assayed by measuring the respiratory control index (refer to the succeeding texts) expressed by the ratio state 3/state 4 respiration [17]. The values obtained were always higher than five with succinate and ADP and not lower than nine with succinate+ the uncoupler carbonyl cyanide 4-(trifluoro-methoxy) phenylhydrazone (FCCP). P/O ratios constantly had a value ranging between 1.9 and 2.1 with freshly isolated mitochondria.

**Mitochondrial Membrane Potential (ΔΨm) Monitoring**

ΔΨm variations were followed fluorimetrically, according to the safranine O method [18,19], employing a Cary-Varian Eclipse spectrofluorimeter set at the excitation and emission wavelengths of 520 and 580 nm, respectively. Typically, to a cuvette containing 3.0 ml of 20 or 125 mM KCl medium, RLM (1 mg/ml), 6 μM rotenone, and 10 μM safranine O were added. After 3 min of equilibration, required to dissipate the ΔΨm generated by endogenous substrates, the instrument was zeroed and 5 mM Tris-succinate was added, followed by 1, 5 or 10 mM of either 1, 2, 3, or 4. If necessary, ΔΨm dissipation was completed with 1 mM KCN or 1 mM FCCP.

**Oxygen Consumption Measurements**

Oxygen consumption was determined using a Clark electrode (Rank Bros., Cambridge, UK, and Hansatech Instruments, UK) housed in a closed thermostated chamber at 25 °C and equipped with a magnetic flea for constant stirring. Freshly isolated mitochondria were incubated, at the concentration of 0.5–1.5 mg protein/ml, in the 20 mM KCl medium, supplemented with 1 mM EDTA, 6 μM rotenone, 2 mM potassium phosphate (Pj), and with 5 mM MgCl2 for respiratory control index determination only. The respiration started with the addition of 5 mM potassium succinate; as appropriate, the following additions were then made: 250 μM ADP followed by 1.5 μM FCCP after exhaustion of the former or 10 mM of either 1, 2, 3 or 4.

**Mitochondrial Swelling**

Mitochondria (3 mg protein) were incubated in a spectrophotometric cuvette at 25 °C in 3 ml 20 mM KCl medium containing 3 μM rotenone and 10 mM of 1, 2, 3, or 4. The absorbance changes were determined at 650 nm [5,20] with an AMINCO DW-2 double wavelength spectrophotometer digitalized by Olis (On Line Instruments Systems, Bogart, GA, USA), so as to rule out any possible interference by redox transition of mitochondrial cytochromes. At the time indicated in Figure 4, 2 mM Pi, and 5 mM succinate were sequentially added.
Cation Binding Studies

The stability constants ($K_s$), relative to the complexation equilibrium

$$L + M^+ \leftrightarrow LM^+$$

($L = 1-4; M^+ = Na^+, K^+, or Cs^+$), quantitatively expressed by

$$K_s = \frac{[LM^+]}{[L][M^+]^\alpha}$$

([M$^+$], [L], and [M$^+$]) being the equilibrium concentrations of the complex, the free ligand, and the free cation, respectively, were estimated by spectrophotometric titrations of the ligand with solutions of the metal chloride salt in methanol, using a Shimadzu UV-2401PC spectrophotometer set at the free ligand maximum absorbance (ca. 203 nm) at 25.0 ± 0.1 °C cuvette compartment.

$K^+$ and Cs$^+$ complexes

The typical experiment was as follows: a solution of the ligand (0.82 × 10$^{-4}$–1.50 × 10$^{-4}$ M) in methanol, contained in a cuvette, was titrated with a concentrated stock solution of the chloride salt in methanol (0.048–0.056 M), added by a precision syringe. After each addition, attainment of equilibrium was checked by observation of no change in the spectra after several minutes. The absorbance values recorded were corrected for dilution by calculation and plotted against metal ion/ligand total concentration ratio ($C_L/C_M$), and $K_s$ were then estimated by nonlinear least square analysis ($R^2 \geq 0.998$), according to the equation (derived for 1:1 complexes):

$$Y = Y_0 + \frac{Y_m - Y_0}{2} \left(1 + \frac{C_M}{C_L} + \frac{1}{K_s C_M} \right) \left(\sqrt{\left(1 + \frac{C_M}{C_L} + \frac{1}{K_s C_M}\right)^2 - 4 \frac{C_M}{C_L}}\right)$$

where $Y_0$, $Y$, and $Y_m$ are the absorbance values before, during, and after the titration, respectively [21]. $K_s$ values from three independent experiments were averaged.

$Na^+$ complexes

The procedure described in the preceding texts, using solutions 1.00 × 10$^{-4}$–1.50 × 10$^{-4}$ M of the ligands and 0.0159–0.0268 M of NaCl in methanol, was substantially followed. In this case, however, titrations were run in a volumetric flask, because of the higher concentration of salt required for sizeable change in UV spectra, as a consequence of the lower $K_s$ values of (Hy) VLM–Na$^+$ complexes. This allowed to estimate $K_s$ more conveniently by means of linear Benesi–Hildebrand plots [21], according to the following equation:

$$\frac{Y_D}{Y_0} = \frac{\alpha}{K_s C_M} + \alpha$$

with $\alpha$ being a constant [21]. Plots of $Y_D/(Y_0 + Y_m)$ versus $1/C_M$ gave straight lines ($R^2 \geq 0.998$), from which $K_s$ was calculated as “intercept/slope” ratio. $K_s$ values from three independent experiments were averaged.

Ethics Statement

Animal experiments were performed in compliance with Art. 9 del Decreto Legislativo 116/92 on animal welfare and approved by the Italian Health Department.

Results and Discussion

Effect on $\Delta \Psi_m$

On the ground of accumulated knowledge about VLM ability to induce cellular apoptosis through dissipation of $\Delta \Psi_m$ [5–7], we set out to compare the efficiency of analogs 2–4 relative to VLM in collapsing the $\Delta \Psi_m$ of RLM. Keeping in mind that drugs with IC$_{50}$ values in the low nanomolar appear best tailored for successful targeted cytotoxic activity [12], all measurements were purposefully performed with compounds 1–4 in the 1–10 nM range; $\Delta \Psi_m$ variations were easily followed fluorimetrically with safranine O as a probe [18,19]. Results are illustrated in Figure 2.

Initially, we monitored the $\Delta \Psi_m$ variations in a 20 mM KCl medium, which is typical for sizeable effects [22]. Under these conditions, the fluorimetric traces of Figure 2(A) reveal that addition of 1 mM 1–4 to succinate-energized RLM induced a $\Delta \Psi_m$ loss, the rate and extent of which appears characteristic for each macrocycle, hinting that the $K^+$ influx catalyzed by compounds 1–4 occurs at different rates. It is seen that VLM still remains the most potent depolarizing agent of the four analogs, causing the largest loss of $\Delta \Psi_m$ and at the highest rate of 57 ± 5 fluorescence units min$^{-1}$ (Figure 2(A), trace ‘1’). It is noteworthy that

![Figure 2](image-url)
the relative efficiency of each HyVLM appears to be strictly depending upon the site of hydroxylation. Specifically, position of a hydroxyl group on D-Val or L-Val residue (analog 3 and 4, respectively) reduces the rate to 32 ± 2 and to 22 ± 1, respectively, as well as the extent of ΔΨm loss (Figure 2(A), traces ‘3’ and ‘4’, respectively); more so when placing OH at the side chain of a D-Hyi residue, in which case the corresponding analog 2 seems hardly able to induce ΔΨm dissipation, the depolarization rate being only 9 ± 1 (Figure 2(A), trace ‘2’). In all cases, the collapse of ΔΨm becomes completed upon addition of either the respiratory inhibitor KCN or of the uncoupler FCCP, indicating that the relatively low concentration of (Hy)VLM and K+ used allowed respiring mitochondria to maintain a residual electrochemical gradient, because of an incomplete (Hy)VLM-dependent K+ recycling.

Figure 2(B) shows that the effect of increasing both the (Hy) VLM concentration (from 1 to 10 nM) and the [K+] of the medium (from 20 to 125 mM, i.e. close to the intracellular concentration) is to produce a more rapid and extensive drop in ΔΨm [22], although the different order of potency of the various analogs, i.e. 1 > 3 ≥ 4 > 2, is overall maintained. We find that the collapse of ΔΨm is generally complete when the rate of fluorescence variation is over ~200 fluorescence units min⁻¹, that is, in the 125 mM KCl medium with ≥5 mM 1, 3, and 4, but only with ≥10 mM 2. It is noteworthy that replacement of KCl with 20 mM NaCl in all the aforementioned experiments (not shown) did not result in any appreciable ΔΨm decrease, either with VLM or with HyVLMs, suggesting that the latter (similarly to the former [23]) are not able to shuttle Na⁺ across membranes.

**Effect on Mitochondrial Respiration and Swelling**

Other relevant hints supporting the aforementioned facts came from the evaluation of the efficiency of each HyVLM in accelerating mitochondrial respiration and volume change (swelling), in comparison with VLM [5,22]. It is well established that to compensate for the VLM-induced dissipation of ΔΨm (setup by respiration), mitochondria respond by speeding up their respiratory rate and oxygen uptake, in a dose-dependent manner [24], so that the faster the respiration, the more efficient is the depolarizing agent under similar conditions. Concomitantly, mitochondria accelerate their swelling rate, as a result of K⁺ influx and of an increased Pi uptake [5,22]. As an example, Figures 3 and 4 show the profiles of stimulated mitochondrial respiration and swelling by 10 nM 1–4 in 20 mM KCl medium.

The polarographic traces of Figure 3 show that state 4 respiratory rate of succinate-respiring mitochondria was remarkably increased on addition of VLM or HyVLMs. VLM, however, displays the highest efficiency in stimulating respiration, expressing a rate of 83 ± 7 nmol O₂ min⁻¹ mg protein⁻¹ (Figure 3, 1 trace), while the rates induced by HyVLMs decrease to 74 ± 5, 64 ± 5, and to 53 ± 4 for 3, 4, and 2, respectively (Figure 3); this order parallels the efficiency displayed by compounds 1–4 in dissipating ΔΨm under the same experimental conditions (Figure 2(B)). We could also verify that respiration is not accelerated in a K⁺-free medium nor in a Na⁺-containing medium only.

Figure 4 illustrates mitochondrial volume changes both in the absence (control experiment) and in the presence of VLM or of analogs 2–4, assessed spectrophotometrically by following the absorbance variations at 650 nm [5,20]. In the absence of any valinomycins, the volume increase is the result of solute uptake supported by succinate oxidation, expressed by a decrease in the absorbance of the mitochondrial suspension (Figure 4, mean value ± SD).
‘control’ trace) [5]. This is truly a reversible energy-dependent swelling, being supported by the energy of the electrochemical proton gradient generated by the activity of the respiratory chain [5], so that its inhibition by antimycin A (AA) addition allows mitochondria to recover their original volume (Figure 4, control trace).

On the other hand, the presence of 10 nM VLM or HyVLMs induces mitochondria to swell faster and more extensively (Figure 4, traces 1–4). Even on a qualitative basis, it can be clearly appreciated that the amplitude and rates of volume change induced by 1–4 increase in the order of $1 > 3 ≈ 4 > 2$, thus faithfully matching the results from rates of $ΔΨ_m$ collapse and $O_2$ consumption (Figures 2 and 3). Because the incubation medium contained 20 mM KCl, the acceleration of mitochondrial swelling upon addition of 1–4 could be ascribed to a high rate of $K^+$ influx, as well as to an increased uptake of $P_i$ which recalls an additional volume of water inside the matrix [5]. Indeed, if the incubation medium was depleted both of $P_i$ and/or $K^+$, the swelling supported by both succinate oxidation per se and by (Hy)VLMs could be no more detectable. Note that the intense activity of the respiratory chain makes the rate of $O_2$ uptake (supported by succinate oxidation) prevail over the rate of atmospheric $O_2$ dissolving into mitochondrial suspensions until anaerobiosis was attained. In line with data from $O_2$ consumption rates (Figure 3), the faster the respiration, the earlier anaerobiosis takes place (Figure 4). This situation is a mimic of the presence of a respiratory inhibitor (e.g. AA; control trace), causing mitochondrial initial volume to be fully restored, as indicated by the increase in the absorbance (Figure 4). This suggests that, similarly to what occurs in the presence of VLM [5], the hydroxyl analogs 2–4 do not cause a high amplitude and irreversible swelling but a fully recoverable increase of mitochondrial volume.

**Effect on Cation Binding**

On the basis of all the previous texts said, it seems that hydroxylation has altered the exceptional $K^+$ ionophore properties of VLM, reflected on the reduced bioactivity of its hydroxyl analogs 2–4. In order to learn whether this effect might be because of variations in the ion binding properties, we undertook to estimate the stability constants ($K_s$) associated with the binding of HyVLMs with selected alkali metal ions ($Na^+$, $K^+$, and $Cs^+$). Reportedly, VLM forms 1 : 1 complexes with such cations [1,2,25–27], although nature has tailored the central cavity of the macrocycle to specifically host a ‘naked’ potassium ion [1,2,25–27]. This potassium specificity is reflected on the high potassium to sodium selectivity, the $K^+/Na^+$ complexing ratio being ca. 10$^4$–10$^5$. On the other hand, with $Cs^+$ the $K_s$ falls to approximately 1/3 of the VLM–$K^+$ stability constant [2], because of unfavorable steric hindrances ensuing the coordination of the larger Cs$^+$.

Assuming that a 1 : 1 complex between ligands 2–4 and the metal ion is formed, the various $K_s$ were assessed spectrophotometrically by measuring the change in the ligand UV maximum absorbance (ca. 203 nm) during titration with the metal ion in methanol at 25.0 ± 0.1 °C [1,2]. Results in the decade logarithmic forms (Log $K_s$), along with relevant literature Log $K_s$ values for VLM complexes under similar conditions [2], are listed in Table 1.

From data in Table 1, it follows that the stability constants of VLM–$M^+$ complexes determined herein match very closely the values reported elsewhere [2]. On the other hand, the $K_s$ of HyVLM–$M^+$ complexes are very similar to those of VLM; the ease of binding follows the same order of $Na^+ << Cs^+ < K^+$, suggesting that the size of the macrocycle internal cavity still governs the cation selectivity. Additionally, the assumption that 1 : 1 complexes are formed is mathematically verified, resulting from the least square fitting of data.

Noticeably, HyVLMs seem to show a higher propensity than 1 to complex with $Na^+$ under the experimental conditions adopted, the corresponding $K_s$ of HyVLM complexes with $Na^+$ being about fivefold higher than with 1 in all cases (Table 1). The obvious consequence of this is that such hydroxyl derivatives experience a sensible drop in the $K^+/Na^+$ complexing selectivity, that is from ca. 3000 for the parent VLM to ca. 350 for, e.g. the hydroxyl derivative 3 (Table 1). In spite of this, however, we have mentioned previously that $Na^+$ is not transported by HyVLMs across the mitochondrial membranes.

Because the stability constants of each HyVLM complex with $K^+$ and $Cs^+$ are not affected by the presence of hydroxyl sites (Table 1), it is reasonable to postulate that the rigid tertiary structure featuring the VLM–$K^+$ and VLM–$Cs^+$ complexes [25–27] is substantially maintained by analogs 2–4 on binding such ions. This assumption leads to a cascade of reasonable speculations, which might provide a tentative rationale to our findings. First, the reduced bioactivity of HyVLMs should not be ascribed to a reduced affinity of such ligands for $K^+$. Second, the hydroxyl sites should not interfere with coordination of such ions, most likely because the OH groups are away from the cavity wherein the ions are housed. Indeed, it is well established that the rigid

<table>
<thead>
<tr>
<th>M$^+$</th>
<th>1$^b$</th>
<th>2</th>
<th>3</th>
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<tr>
<td>$K^+$</td>
<td>4.90$^c$</td>
<td>4.60 ± 0.01</td>
<td>4.53 ± 0.02</td>
<td>4.44 ± 0.02</td>
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<td>4.43$^d$</td>
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<td>&gt;3.9$^e$</td>
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<tr>
<td>$Cs^+$</td>
<td>4.41$^c$</td>
<td>4.18 ± 0.05</td>
<td>4.08 ± 0.04</td>
<td>4.15 ± 0.03</td>
</tr>
<tr>
<td>$Na^+$</td>
<td>0.67$^c$</td>
<td>1.15 ± 0.01</td>
<td>1.86 ± 0.02</td>
<td>1.90 ± 0.03</td>
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$^{a}$Mean values ± SD (n = 3).

$^{b}$Literature values.

$^{c}$Spectrophotometry.

$^{d}$Conductivity.

$^{e}$Potentiometry.
conformation of VLM–K⁺ and VLM–Cs⁺ complexes in solution resembles a hollow cylinder, in which the isopropyl side chains point outward, forming the hydrophobic outer layer [25–27]. In particular, the isopropyl moieties of 0-Val and L-Val residues are spatially arranged in a way that they screen the entrances to the cavity, whereas those of 0-Hyi residues are placed around the equatorial belt (Figure 5).

Consequently, in folding around the metal ion, macrocycles 2–4 should force the hydroxyl groups to project into the molecular surface of the complex, thus subtly modifying its characteristics and hence the HyVLMs properties of transmembrane K⁺ transport. This is likely to slow down the HyVLMs-catalyzed K⁺ transport, so that we see that mitochondria are able to counteract the HyVLMs action, especially in low [K⁺] medium (Figure 2). Interestingly, one may envisage a correlation between the extent of bioactivity loss and the spatial orientation of the various isopropyl moieties bearing the OH group. According to Figure 5, the approximately symmetrical organization of the 0-Val and L-Val isopropyl groups around the cylinder-like VLM–K⁺ structure (Figure 5) is such that, upon hydroxylation, the VLM analogs 3 and 4 display almost the same levels of bioactivity (Figures 2–4). Hydroxylation at the 0-Hyi side chain, which is placed around the equatorial surface (Figure 5), should instead bring a major contribution in the modification of the surface properties of the K⁺ complex of analog 2, so that the latter undergoes a more severe loss of bioactivity.

Conclusions

The bioactivity of a three-entity library of hydroxyl valinomycins, carrying the OH group at the side chain of a 0-Hyi, 0-Val, or L-Val residue has been assessed on the basis of their relative efficiency to alter bioenergetics parameters of isolated RLM, the target of VLM. The key aspects of our findings can be summarized as follows:

• Hydroxylation reduces the bioactivity of VLM.

• The extent of reduced bioactivity appears to depend upon the molecular site involved in the hydroxylation, so that situation of the OH group at the side chain of 0-Val and L-Val residues leads to derivatives (3, 4), which more closely resemble the parent VLM behavior. Whereas, the drop in bioactivity is exceptionally marked when hydroxylation involves the side chain of a 0-Hyi residue (analog 2).

• The OH group does not impact the ability of analogs 2–4 to complex with Na⁺, K⁺, and Cs⁺; the stability constants of complexes being very similar to those of VLM, except for a modest increase in stability of Na⁺ complexes. Nonetheless, Na⁺ is not transported by HyVLMs across mitochondrial membranes.

These findings suggest that incorporation of the hydroxyl may have actually introduced sensible variations in the VLM properties of K⁺ transporting across mitochondrial membranes. We believe that the extent of bioactivity loss observed reflects the degree of OH group exposure to the molecular surface, determined ultimately by the relative spatial arrangement of the isopropyl groups within the K⁺ complex conformation. This brings to light the different contribution of the various isopropyl moieties to the exceptional efficiency of the VLM-catalyzed K⁺ transport across membranes.

From a practical standpoint, the results presented herein indicate that HyVLMs overall retain the basic aspects of VLM bioactivity, a key requirement that, in principle, makes them amenable to ligand targeting. Furthermore, the site-selective hydroxylation of VLM could be an expedient route to finely tune its bioactivity, bringing thus a major flexibility in the rational design of VLM-based conjugates.

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